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Mutation Drift and Repertoire Shift in the Maturation of the Immune Response

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INTRODUCTION

The hallmark of the immune response is its specificity and the specificity is directly correlated with the affinity of the antigen-antibody interaction. The requirement for high affinity antibodies may be more important than specificity alone, since antibodies are designed to detect soluble antigens which are sometimes capable of inflicting great harm at very low concentrations (e.g. toxins). This may not be required by, or may even be a disadvantage to T-cell responses where the affinity for the ligand involves interactions of the T-cell receptor not only with antigen, but also with other molecules, e.g. those involved in MHC restriction (Yague et al. 1985, Dembic et al. 1986). T cells therefore may not have developed the equivalent of the elaborate mechanism which is the object of this paper. During the course of an antigen-specific immune response, the affinity of the serum increases with time, a phenomenon commonly referred to as maturation of the response (Jerne 1951, Siskind & Benaceraff 1969). Such a maturation results from specific alterations of the structure of the antibody molecules (Steiner & Eisen 1967). What is the precise nature of these alterations, which are the root of the production of high affinity antibodies?

There is no doubt that somatic mutation contributes to antibody diversity (Weigert et al. 1970, Bernard et al. 1978, Griffiths et al. 1984). There are many reasons to believe that a mechanism of hypermutation operates within restricted stretches of the DNA to further diversify the genes encoding the antibody molecules (Kim et al. 1981, Gearhart & Bogenhagen 1983). This mutational drift is, however, not the full extent of the change. Major changes in the antibody structures involved result from a shift in the antigen-specific B-cell repertoire over the course of the immune response. In the primary response the most frequent B-cell clones already expressing antibody molecules with a relatively

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high affinity for the antigen are likely to become initially the dominating population. Some of these cells differentiate into terminal plasma cells, others into long-living memory cells. It is not clear how random the frequency of expression of the different germ line gene combinations is (Yancopoulos et al. 1984, Wood & Coleclough 1984), nor what determines the fate of the individual B cell. In any event, it is conceivable that the repertoire of the primary response plasma cells differs from the repertoire of the memory cells which will be stimulated by further contact with antigen.

Maturation is the result of selection and specific expansion of B-cell clones expressing high affinity antibody molecules. However, the overall process is considerably more complex than this statement implies. This is shown by the fact that, in memory responses, clones appear which have as low affinities as those of primary responses. These clones usually express new V(D)J germ line gene combinations, and may constitute an important source from which new high affinity antibodies arise. This change in the repertoire of the antigen-specific B cells is likely to be connected with cellular properties (Freitas et al. 1986) including circulation pathways, cell dynamics, differential properties of virgin and memory cells, compartmentalization, and other cellular interactions such as those described by the network hypothesis (Jerne 1974). This is a remarkable property of the immune system, which implies selective proliferation, not only of cells expressing antibodies of improved affinity, but also a capacity to encourage the expression of alternative possibilities.

THE IMMUNE RESPONSE TO THE HAPTEN 2-PHENYL-5-OXAZOLONE

In order to study the maturation of an antigen-specific immune response, mice were immunized with the hapten 2-phenyl-5-oxazolone (phOx) coupled to the carrier chicken serum albumin (CSA) and spleen cells of such mice were fused to the non-secretor line NSO (Kaartinen et al. 1983a) at various stages of the immune response (Fig. 1). Hybridoma lines secreting antibodies with specificity for the hapten phOx were selected, cloned and grown up for preparation of mRNA. Synthetic oligonucleotides complementary to sequences in the constant and variable regions of H- and L-chains were used as specific primers and the sequence of the mRNA determined as described (Hamlyn et al. 1978, Griffiths & Milstein 1985). In a first set of experiments, mice were immunized intraperitoneally with 30 μg alum precipitated phOx-CSA and spleen cells fused 7 or 14 d after the primary injection (Fig. 1). Sequence analysis of d 7 response antibodies showed that the majority of the hybridoma lines secreted identical or almost identical antibody molecules (Kaartinen et al. 1983a, Kaartinen et al. 1983b). Most of the H- and L-chains had utilized a particular combination of V-germline genes which were named V_κ-Oxl and V_H-Oxl respectively. In addition, strong restrictions in the D/J regions were found. In the L-chains, V_κ-Oxl was joined to

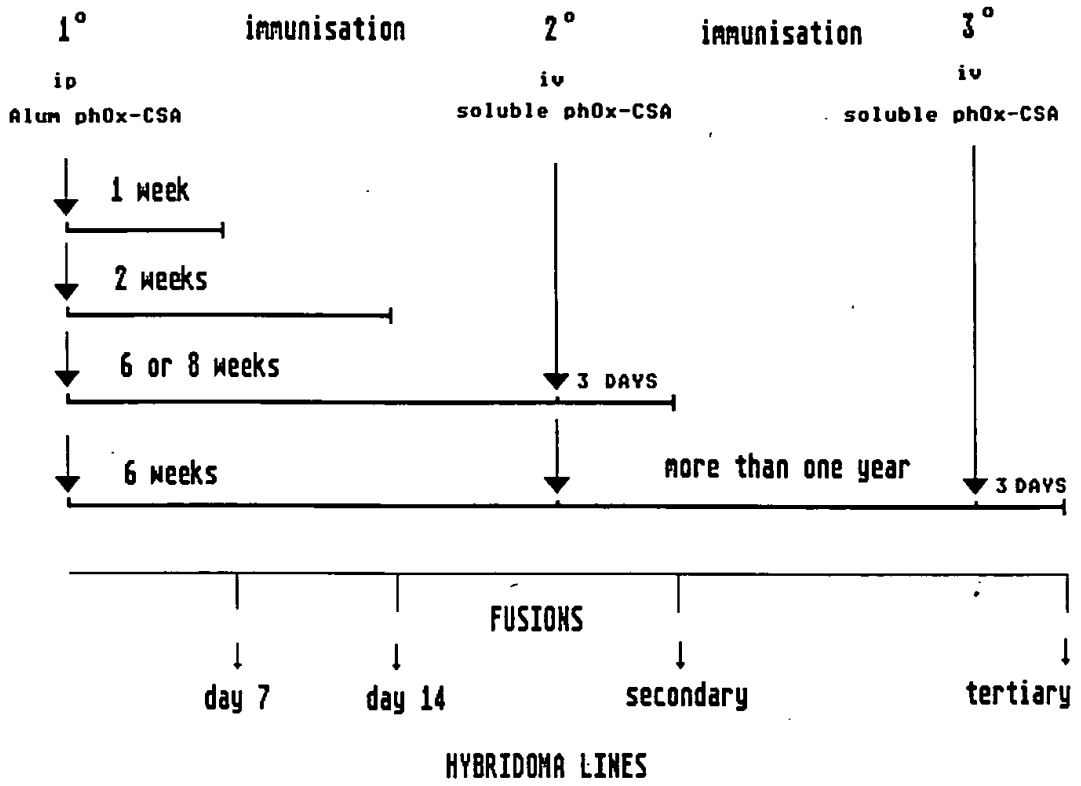

Figure 1. Protocol used for the analysis of the onset and maturation of the immune response to phOx-CSA.

$J_\kappa 5$, which encodes Leu at position 96. In the only case where a different J segment ($J_\kappa 4$) was expressed, the joining residue 96 was not the germline gene residue coded by $J_\kappa 4$, but a leucine generated as a V-J joining variant. The V_H-Oxl heavy chain showed conservation not only in the length of the D-J region (in all cases 16 amino acids) but also in the amino acid sequence. The first residue of the D-segment was always Asp, the third residue Gly, and only the middle residue showed variability.

One week later, 14 d after the primary injection, most antibodies still expressed similar, but no longer identical sequences, suggesting a high degree of somatic mutation (Fig. 2). These changes were associated with an increase in affinity for the hapten (Griffiths et al. 1984).

To study the secondary response, mice were rested after the primary injection for 6 or 8 wk, and then boosted with an intravenous injection of soluble antigen (phOx-CSA). Three days after the secondary immunization, spleen cells were fused, antigen-specific hybridoma lines characterized, and again the sequence for the H- and the L-chains of the antibody molecules determined. In the secondary response, less than 20% of the hybridoma lines secreted antibody molecules which showed the V_H-/V_κ-Oxl combination predominantly found in the primary

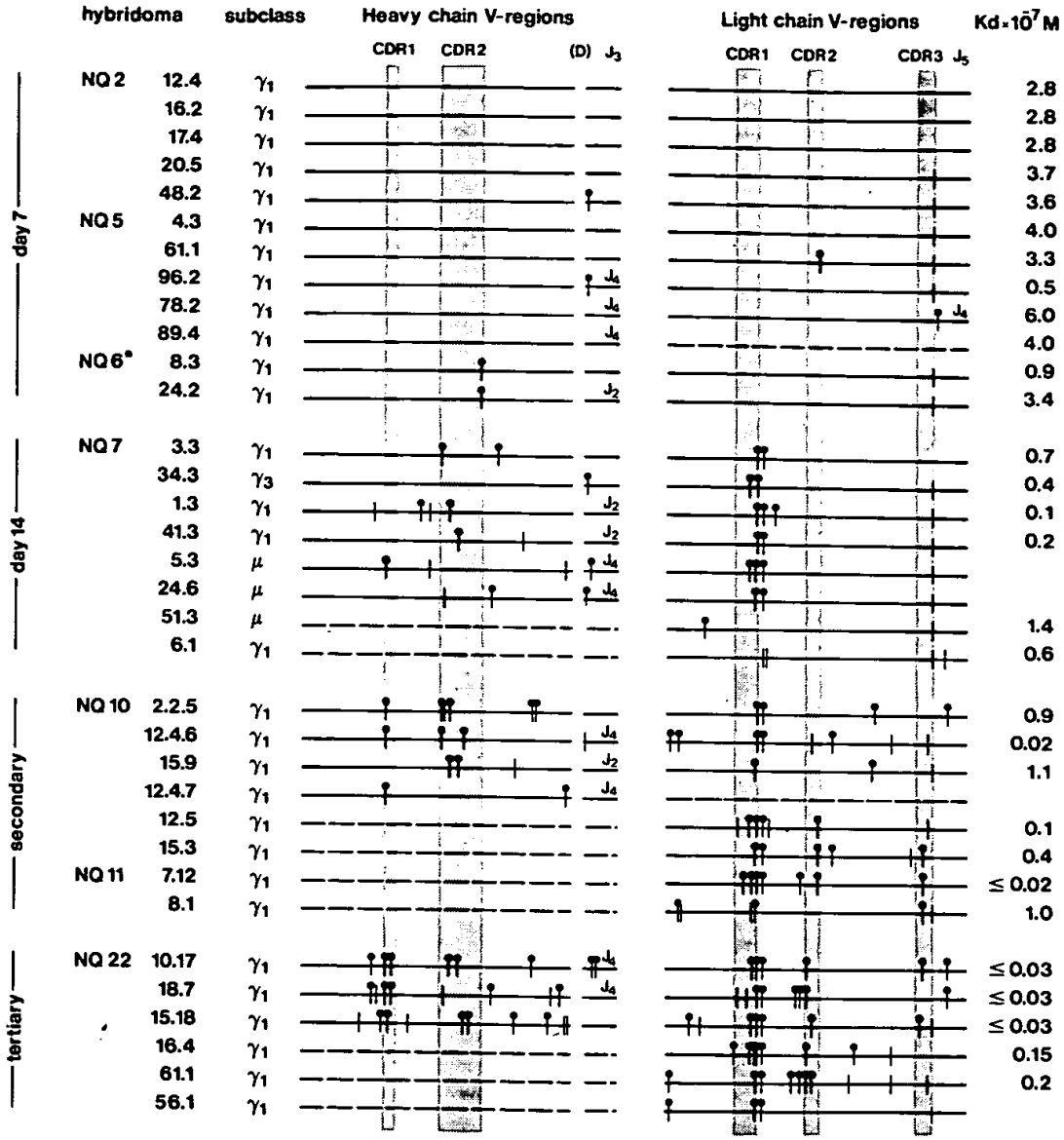

Figure 2. Diagrammatic comparison of mRNA sequences from phOx-specific hybridomas. Only sequences considered to be derived from V_κ-Ox1 or V_H-Ox1 germline genes are compared in detail. Unbroken lines denote sequence identity, broken lines indicate sequences derived from different germline genes. A vertical bar shows the relative position of codons containing nucleotide differences. A black circle indicates that these changes predict an amino acid difference at this position. Complementarity-determining regions (CDR-1, -2, -3) have been marked, as have the D- and J-regions. D-region sequences have not been included. They usually code for Asp-X-Gly. BALB/c mice were used in all cases except in NQ6*, where a DBA/2 mouse was used. The V_H-Ox1 difference between DBA/2 and BALB/c is due to polymorphism. The affinity of the antibodies was measured from purified preparations by fluorescence quenching using the hapten phOx-Cap and/or by equilibrium dialysis using [3H]phOx-aminobutyrate. More recent unpublished values (NQ22) were obtained by equilibrium dialysis using the non-purified hybridoma culture supernatants. Data were taken from Kaartinen et al. (1983b), Griffiths et al. (1984), and unpublished data from the authors.

response (Griffiths et al. 1984). Those few hybridoma lines which expressed V_H-/V_κ-Oxl showed even more somatic mutations than the late primary response antibodies, although the number of somatic mutations did not necessarily correlate with a further increase in the affinity for the hapten phOx.

Characteristic of the secondary response was a shift of the frequency of B-cell clones expressing Oxl antibodies towards clones expressing other H-/L-chain combinations (Berek et al. 1985). In the secondary response antibodies, the V_κ-Oxl L-chain was found in association with V_H-segments not observed in the primary response. At the amino acid level these V_H-segments had only about 60% homology with the V_H-Oxl sequence, and belonged to different V_H-gene families, mainly MOPC21 or J558 gene family (Dildrop 1984). It was remarkable that these different H-chains combining with the V_κ-Oxl L-chain showed the same restriction in their D-J regions as found for the Ox1 H-chain. Another group of secondary response antibodies had L-chains different from V_κ-Ox1. In these phOx-specific antibodies, the V_κ-45.1 L-chain was found mainly in combination with H-chains similar to the V11 germline gene (Crews et al. 1981).

In order to look at the tertiary response, mice immunized as described were rested for more than 1 yr, and then boosted with an intravenous injection of soluble phOx-CSA. Three days later, spleen cells were fused and again the sequences of the H- and the L-chain mRNA determined. The majority of the antibody molecules showed H-/L-chain combinations already observed at previous stages of the immune response. The maturation of the tertiary response antibodies was characterized by a further increase in the somatic point mutations (Fig. 2). The analysis showed that a further drift had taken place (Berek & Milstein, manuscript in preparation).

V_κ-Ox1 AND RELATED GERMLINE GENES

The primary d 7 response to the hapten phOx is dominated by antibodies with a particular L-chain, V_κ-Ox1. The sequence was first postulated and later shown to represent a germline gene.

From two different BALB/c germline libraries, 13 different genes hybridizing to a V_κ-Ox1 probe were isolated and characterized (Even et al. 1985). A statistical analysis of the data indicated that the V_κ-Ox1 related gene family contains more than 20 and probably less than 50 genes. The sequences of two of them were identical to mRNA sequences found in the early primary response. One of these germline genes had a sequence identical to the V_κ-Ox1 L-chain mRNAs of the d 7 response. The next most closely related germline gene differed by only six nucleotides, but none of these changes has been found in any of the late response V_κ-Ox1 mRNA sequences. The germline sequences showed a pattern of nucleotide changes different from the mRNA sequences of the high affinity phOx specific

antibodies, thus substantitating the view that the V_κ-Ox1 variants are indeed somatic variants of the V_κ-Ox1 germline gene.

SOMATIC MUTATION AND B-CELL DIFFERENTIATION

Antibody diversity of uncommitted B cells arises by combinatorial usage of V(D)J gene fragments to form functional heavy and light chains, and by junctional diversity generated during the recombination process (Tonegawa 1983). Antigen-stimulated cells show further diversification introduced by somatic point mutations scattered throughout the V-regions. It had previously been postulated (Lederberg 1959) that a specific hypermutation mechanism restricted to the V-region (Brenner & Milstein 1966) is involved in the generation of diversity, leading to the idea that this could be the basic event responsible for the maturation of the immune response. Data obtained with a myeloma line (Cook & Scharff 1977) and also using hybridoma lines obtained from immune spleen cells have suggested values of up to 10^{-3} per base pair per generation (McKean et al. 1984, Sablitzky et al. 1985) for the hypermutation rate.

Whether there are certain stages in the development of the B-lymphocyte in which the proposed hypermutation takes place, or whether this mechanism is an ongoing process diversifying the variable regions throughout B-cell commitment and differentiation remains controversial. If the hypermutation mechanism is activated by the antigenic stimulus, an unstimulated B cell should rarely, if at all, display point mutations in the V-regions. In the pre-B lymphocyte line 18-81, a frequency of somatic mutation of 10^{-5} per base pair per generation has been found (Wabl et al. 1985). This compares with the value of 3.3×10^{-6}/cell/generation for the frequency of electrophoretic mutants of the myeloma protein MOPC21 involving well over 1000 base pairs (Adetugbo et al. 1977). On the other hand, sequence analysis of *in vitro*-activated normal B cells has shown no evidence of somatic mutation (Manser et al. 1984). However, these experiments were designed to detect mutation rates of 10^{-3}, but not of 10^{-5} per base pair per generation.

That the mutation rate of unstimulated or even newly stimulated B cells was not as high at 10^{-3}/base pair/generation was implicit in our analysis of the primary response to the hapten phOx. The antibodies from the d 7 response did not show evidence of somatic mutations in the variable regions of 10 H- and 9 L-chains, except for one point mutation involving a single nucleotide exchange (Kaartinen et al. 1983b). The number of nucleotides sequenced in hybridomas from the d 7 response involved well over 5×10^3 nucleotides, implying an average mutation frequency of 2×10^{-4} per base pair, and an even lower mutation rate when we take into account that the cells originated from a proliferating pool. This calculation thus shows that the average mutation rate cannot be as high as

10^{-3} per base pair per generation, even immediately after primary antigenic stimulation.

The next stage involves a dramatic change (Fig. 2). The late primary response (d 14) variable regions of H- and L-chains showed, on average, 3 nucleotides exchanges per sequence (around 300 nucleotides), i.e. approximately 1% of the nucleotides have mutated (Griffiths et al. 1984). A calculation of real mutation rates is difficult to derive, because of the importance of selective pressure. Antigenic selection is clearly playing a critical role in the process of affinity maturation. Key mutations affecting the affinity for phOx are observed as independently repeated events viz the His-Asn/Gln substitution at residue 34 of the V_κ-Ox1 light chain in all Ox1 antibodies of the d 14 response (Table I). It is, however, most unlikely that every mutation observed gave selective advantage to the relevant clone either by antigen, or by idiotypic interactions. We notice, for instance, that there are 10 silent substitions in the 8 light and 6 heavy chains of d 14. The frequency of silent and presumably unselected mutants is as much as 2.5×10^{-3} per base pair. If the assumption is valid that silent mutations (or at least some of them) are unselected, that observation alone strongly suggests that there has been an increase in mutation rate after the primary stimulus has taken place.

In the d 7 analysis, there were no silent mutants, and therefore the frequency of accumulated mutants was $< 2 \times 10^{-4}$. The difference between $< 2 \times 10^{-4}$ at d 7 and 2.5×10^{-3} at d 14 could be accounted for by an increase in the rate of somatic mutation or a change in the number of generations of the growing population. The increase in frequency of unselected mutants in a cell population of constant size is (Stainer 1977):

$$\text{frequency} = \frac{\text{mutation rate} \times \text{number of generations}}{0.69}$$

TABLE I

Somatic mutations in the L-chains of d 14 V_κ-Ox1/V_H-Ox1 antibodies

	V_κ-Ox1							V_H-Ox1	
	31		34	35	36	37	40		
Antibody	S		H	W	Y	Q	S	D	J_H
	AGU	...	CAC	UGG	UAC	CAG	... UCA		
NQ7/ 5.3	–C–	...	A– –	– – –	C– –	– – –	... – – –	GAU CAU GGG	4
34.3	–C–	...	A– –	– – –	– – –	– – –	... – – –	– – – AGC – –A	3
1.3	– – –	...	A– –	– – –	–U–	– – –	... A– –	– – – –GG – –U	2
41.3	– – –	...	A– –	– – –	–U–	– – –	... – – –	– –C – –C – – –	2
3.3	– – –	...	– –G	– – –	–U–	– – –	... – – –	– – – –C– – –	3
24.6	– – –	...	– –G	– – –	–U–	– – –	... – – –	– –A GGG – – –	4

Notice that somatic mutants accumulate in CDR1–FRW2 of V_κ-Ox1 L-chains. A palindrome (residues 34–37) is shown underlined. Data are from Griffiths et al. (1984).

Therefore either the mutation rate, the number of generations, or (more likely) a combination of both, has increased by a factor of more than 10. To put it in better perspective, it is hard to imagine that there were only two generations involved in the first 7 d and 23 generations in the second period of 7 d, with a constant rate of 10^{-4}/base pair/generation for silent mutations. It is more realistic to imagine five generations at d 7 and a further 10 generations at d 14, with an average increase of the rate of silent mutations in the proliferating population from less than 3×10^{-5} to 1.6×10^{-4}/base pair/generation. If silent mutants represent $\frac{1}{3}$ of the total mutants, the overall mutation rate at d 14 would have been around 0.5×10^{-3} per base pair/generation.

It therefore appears that the onset of the maturation process through hypermutation and selection takes place at the time when germinal centers are being organized in the spleen, and these events may not be unrelated (MacLennan & Gray 1985). Hypermutation and selection seem also to be a driving force of maturation at later stages of antigenic stimulation. This is particularly noticeable in the analysis of the anti-phOx secondary and tertiary responses. Certain V-regions, V_H-Ox1, V_H-M21 (MOPC 21-like), V_κ-Ox1 and V_κ-45.1, have repeatedly been found in phOx-specific antibodies. A comparison of sequences obtained at different stages of the immune response shows that, as the response matures, the number of somatic mutations continuously increases. Such results support the conclusion that hypermutation also occurs following repeated antigenic stimulus. The large increase in somatic mutants, however, is not observed in anti-phOx antibody L-chains using the V_κ-45.1 gene segment (Fig. 3). An unexpectedly low number of somatic mutations in λ light chains derived from a secondary response to the hapten NP has also been reported (Cumano & Rajewsky 1986). Perhaps the hypermutation mechanism is not equally active on all V-genes.

SOMATIC MUTATION AND HOT SPOTS

Hypermutation seems to be a enzymatic process specifically acting on the rearranged V-gene independent of whether this gene is translated into a functional H- or L-chain or whether it is on the non-active chromosome (Pech et al. 1981). Even an unrelated sequence (c-myc), when accidentally located on the V-segment position through a chromosomal translocation, has been found to be hypermutated (Rabbitts et al. 1984). Whereas mutations could be found in the flanking sequences adjacent to the rearranged V-gene and/or the J-segment, somatic mutations were rarely detected in the constant regions of myeloma cells (Gearhart & Bogenhagen 1983, Secher et al. 1977). In unpublished experiments performed in collaboration with G. Griffiths, we searched among the hypermutated V_κ-Ox1 chains of d 14 and of the secondary response phOx antibodies for C-region mutants. Hybridomas which included multiple mutations in the V_κ-region did not show any changes in the C_κ-segments sequenced. Therefore the

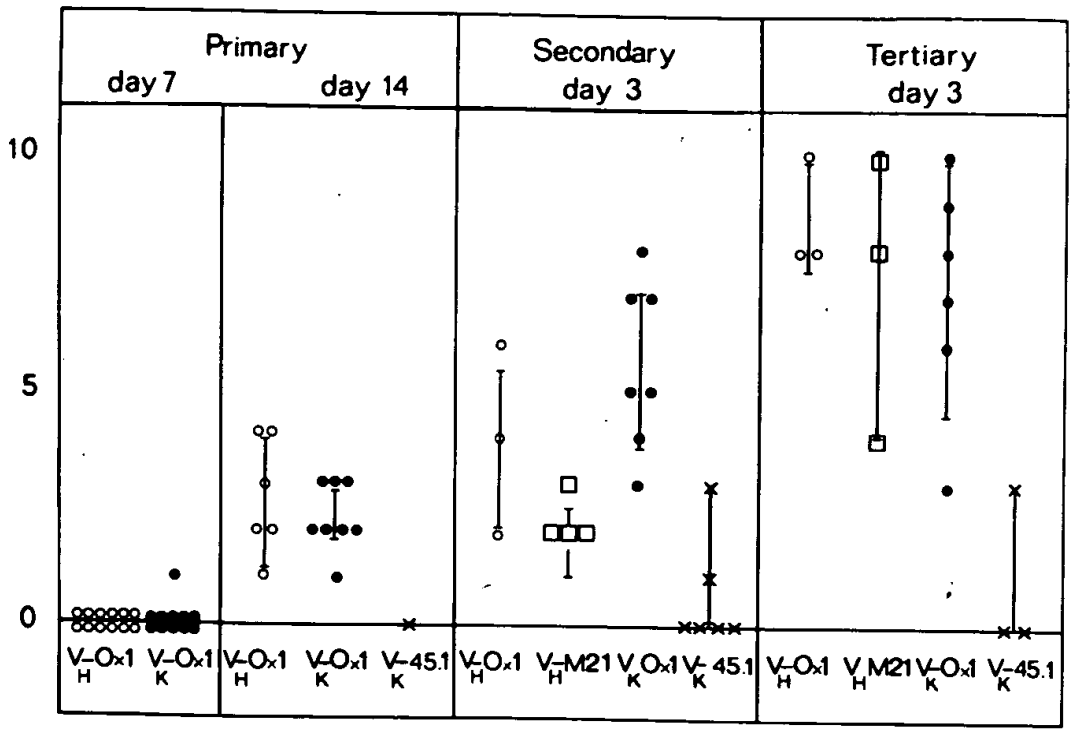

Figure 3. Accumulation of somatic mutations with time after immunization. Number of somatic mutations for primary (d 7 and d 14), secondary and tertiary response antibodies are shown for the H-chains V_H-Ox1 and the MOPC-21 like (V_H-M21) and for the L-chains V_κ-Ox1 and V_κ-45.1.

hypermutational mechanism, as postulated by Brenner & Milstein (1966), seems to be restricted to a specific segment of the DNA including the V-region. Very little else is known about the hypermutational mechanism itself. The high number of changes found in the H- and L-chains of the phOx-specific antibodies enable us to analyze the nature of the somatic mutations in more detail.

It is commonly observed that the same somatic mutant appears in independent hybridoma lines derived from a single immunized mouse. This has often been attributed to divergent variants of a single stimulated clone (McKean et al. 1984, Sablitzky et al. 1985, Clarke et al. 1985, Cleary et al. 1986). This type of repeated variant gives information on the natural history of the clonal diversification during an immune response. However, identical mutations are also observed in antibodies derived from different animals or in hybridoma lines which, based on the differences in germline gene fragments utilized to express the antibody, are considered to be of independent clonal origin. Fig. 4 shows that the number of such independent repeats is considerable. Most of them involve an amino acid substitution (expressed mutation). They may affect antibody structure and therefore could be the result of antigenic selection (parallel or convergent diversifi-

a) V_κ-Ox1

	2	5	26	31	32	33	34	36	37	49	50	55	60	75	81	92	93	94	100	
	Ile	Thr	Ser	Ser	Tyr	Met	His	Tyr	Gln	Tyr	Asp	Val	Val	Ser	Glu	Ser	Ser	Asn	Val	
	AUU	ACC	AGC	AGU	UAC	AUG	CAC	UAC	CAG	UAU	GAC	GCU	GCU	AGC	GAA	AGU	AGU	AAC	GCU	
NQ5.61.1.2	---	---	---	---	---	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---	
NQ 7. 1.3	---	---	---	---	---	---	A--	-U-	---	---	---	---	---	---	---	---	---	---	---	
3.3	---	---	---	---	---	---	--G	-U-	---	---	---	---	---	---	---	---	---	---	---	
5.3	---	---	---	-C-	---	---	A--	*--	---	---	---	---	---	---	---	---	---	---	---	
6.1	---	---	---	---	---	---	---	--*	--A	---	---	---	---	---	---	-C*	---	---	---	
24.6	---	---	---	---	---	---	--G	-U-	---	---	---	---	---	---	---	---	---	---	---	
34.3	---	---	---	-C-	---	---	A--	---	---	---	---	---	---	---	---	---	---	---	---	
41.3	---	---	---	---	---	---	A--	-U-	---	---	---	---	---	---	---	---	---	---	---	
NQ10.2.2.5	---	---	---	---	---	---	A--	-U-	---	---	---	---	---	-U-	---	---	---	---	-*-	
12.4.6	-*-	-A-	---	---	---	---	--G	-U-	---	---	---	A--	---	--G	---	---	---	--U	---	
12.5	---	---	--U	--G	---	---	A--	-U-	--A	---	---	U--	---	---	---	---	---	--U	---	¦related
15.3	---	---	---	---	---	---	~--	-~-	---	---	---	~--	C--	---	---	-A-	---	---	---	¦clones#
15.9	---	---	---	---	---	--A	---	---	---	---	---	---	---	---	---	---	---	---	---	
NQ11.7.12	---	---	---	---	---	G--	--G	-U-	---	-C-	---	C--	---	---	---	-C-	---	---	---	
8.1	---	-U*	---	---	--*	---	A--	-U-	---	---	---	---	---	---	---	-A-	---	---	---	
NQ21.12	---	---	---	---	---	---	---	-U-	---	---	---	---	---	---	---	---	---	---	---	
NQ19.2.4	---	---	---	---	-U-	---	--G	---	---	---	C--	---	---	-C-	---	---	G--	--U	---	
22.21	---	---	---	--G	---	---	A--	-U-	---	---	-G-	---	---	---	---	---	---	---	---	
NQ22.10.17	---	---	---	---	---	--U	A--	-U-	---	---	-C-	---	---	---	---	---	G--	---	U--	¦related
18.7	---	---	--U	---	---	---	~--	-~-	---	-U-	-~-	---	---	---	---	---	---	---	A--	¦clones#
15.18	---	---	---	---	---	G--	--G	-U-	---	---	---	-A-	---	---	---	-C-	---	---	---	
16.4	---	---	---	-A-	-U-	---	A--	-U-	---	---	-G-	---	---	---	--G	---	---	---	---	¦related
61.1	G--	---	---	---	---	---	--G	-~-	---	-U-	U--	A--	---	---	--~	---	---	--U	---	¦clones#
56.1	G--	---	---	---	---	---	--G	-U-	---	---	---	---	---	---	---	---	---	---	---	

b) V_H-Ox1

	3	28	31	46	50	53	56	58	70	76	82C	91
	Gln	Ser	Ser	Glu	Val	Ala	Ser	Asn	Ser	Ser	Leu	Tyr
	CAG	UCA	AGC	GAG	GUA	GCU	AGC	AAU	AGC	AGC	CUG	UAC
NQ7. 1.3	---	--C	---	--A	---	A--	---	---	---	---	---	---
3.3	---	---	---	---	U--	---	---	---	-C-	---	---	---
5.3	---	---	-A-	--A	---	---	---	---	---	---	---	--U
41.3	---	---	---	---	---	---	-A-	---	---	---	---	---
NQ10.12.4.6	---	---*	-C-	---	C--	---	---	-G-	---	---	---	---
12.4.7	--A	---	-C-	---	---	---	---	---	---	---	---	-*-
15.9	---	---	---	---	---	A--	-C-	---	---	---	---	---
NQ19.2.4	--U	---	-C-	---	---	U--	---	---	-A-	---	---	---
NQ22.10.17	---	---	-A-	---	---	U--	-C-	---	---	---	---	---
18.7	---	--C	-C-	---	---	---	---	---	---	---	--A	---
15.18	---	---	---	---	---	---	---	-U-	---	--A	---	--U
NQ25. 90	---	---	*--	---	---	C--	---	---	-A-	---	---	---
195	---	---	---	---	---	---	---	-C-	---	---	---	---
NQ29.D7.8	---	---	---	---	---	---	--*	---	---	--G	---	---
D12.4	---	---	-A-	---	---	---	---	---	---	---	--A	---

Figure 4. Positions exhibiting accumulated independent mutations in phOx antibodies. Sequences are derived from nine independent fusions, NQ5, NQ7, NQ10, NQ11, NQ19, NQ21, NQ22, NQ25 and NQ29.

– hybridoma lines from one fusion having the same V-(D)-J rearrangements. All other lines derived from one fusion differed in V-, D- and/or J-segments.

~ – dentical changes in related clones.

* – single silent or expressed mutations. Repeated silent changes are indicated by black background.

In V_κ-Ox1 80% of the changes are at 7.5% of the positions.

In V_H-Ox1 48% of the changes are at 3.8% of the positions.

cation). A good example is the repeated exchange of residue His 34 in the germ line of V_κ-Ox1 to either Asn or Gln (Fig. 4). However, many of the repeated changes involve conservative substitutions. For instance, the Tyr to Phe substitution at position 36 of V_κ-Ox1 or Ser 31 of V_H-Ox1, where the second base of the triplet has mutated in 7 out of 17 mutated sequences, to give either Thr or Asn. Moreover, many of the repeated mutations are silent, and these cannot be attributed to proliferative advantage due to an improved antigenic recognition.

Frequent repeated silent mutations (Fig. 4) point to hot spots of somatic

mutation. Though only 5 out of the 60 (8%) possible point mutations in the CDR3 of V_κ-Ox1 L-chain would give rise to silent mutations, 5 out of the 12 (41%) observed substitutions were silent. Even more surprising is that 4 of them are independent identical point mutations of the third base coding for Asn 94, AAC → AAU. A mutational hot spot within a residue which must be conserved either for structural reasons or for antigenic specificity would result in an accumulation of silent mutations. Hence, in order to maintain asparagine and, presumably with it, the good affinity for the hapten phOx, only silent mutations can be tolerated. On those grounds, we suggest that the occurrence of repeated silent mutations detected in framework positions (see Fig. 4) implies that hot spots can occur not only in CDR- but also in FRW-residues.

We have made a more detailed analysis of the randomness of the distribution of silent mutations. Table II lists all such independent events as they occur in clumps, and compares them to the number which occur in isolation and to the total number of positions where silent mutations could theoretically occur. The data were then subjected to a statistical analysis (Table III). The results of the analysis show that the pattern of repeats of the V_H-Ox1 silent mutants is very unlikely ($p=0.011$) to be a chance observation if the mutants are genuinely random. More dramatic is the analysis of the V_κ-Ox1 silent mutants. The probability of the observed pattern occurring by chance alone is less than 1×10^{-4}, i.e. virtually impossible. We have no reason to exclude the quadruple repeat at position 94 (shown in Fig. 4), but even if the quadruple repeat is ignored, the outcome is unlikely ($p=0.012$). In any event, the combined probability of the two patterns is very low indeed and gives strong support to the idea that within the variable region the silent mutants (and presumably the expressed mutants as well) are not randomly generated.

Although the statistical analysis of the data indicate a non-random distribution of mutations, the differential mutation rate in hot spots could represent only moderate increases in mutation rates as compared with the dramatic difference

TABLE II

Silent mutations in phOx antibodies

	V_H-Ox1	V_κ-Ox1
Possibilities for silent mutations	166	186
Number of silent changes	19	19
Single silent changes	11	9
Two identical silent changes	4 (28, 46, 82C, 91)	3 (26, 37, 81)
Three identical silent changes	–	–
Four identical silent changes	–	1 (94)
Number of mutated sequences analyzed	19	23

The positions at which the repeated silent mutations occur are shown in brackets. See also Fig. 4.

between the V and C segments. Such non-random distribution is likely to be associated with certain DNA sequence peculiarities. The most obvious could be palindromic sequences. A striking example of one such palindrome (underlined in Table I) has been noted in the mutable segment of the V_κ-Ox1, which encompasses

TABLE III

Probability of a pattern of silent mutations

V_H-Ox1 independent mutants

No. possible silent mutations: 166

Observed: 19

Singles	Pairs	Triples	Quadruples	Distinct Changes	Probability
19	0	0	0	19	0.34278*
17	1	0	0	18	0.39605*
15	2	0	0	17	0.18075*
13	3	0	0	16	0.04217*
11	4	0	0	15	0.00545
16	0	1	0	17	0.01596*
14	1	1	0	16	0.01205*
Other combinations					<0.00363

V_κ-Ox1 independent mutants

No. possible silent mutations: 186

Observed: 19

Singles	Pairs	Triples	Quadruples	Distinct Changes	Probability
19	0	0	0	19	0.38618*
17	1	0	0	18	0.39308*
15	2	0	0	17	0.15816*
13	3	0	0	16	0.03256*
16	0	1	0	17	0.01318*
Other combinations					<0.00930*
9	3	0	1	13	0.00000

V_κ-Ox1 independent mutants excluding quadruple repeat at residue 94

No. possible silent mutations: 185

Observed: 15

Singles	Pairs	Triples	Quadruples	Distinct Changes	Probability
15	0	0	0	15	0.55822*
13	1	0	0	14	0.34277*
11	2	0	0	13	0.07772*
9	3	0	0	12	0.00824
12	0	1	0	13	0.00864*
Other combinations					<0.00392

residues 34 and 36 (Milstein et al. 1986). Whether the association of the palindrome with somatic mutations is more than circumstantial remains to be seen.

SOMATIC MUTATION AND THE INCREASE IN AFFINITY

The increase in antibody binding affinity during maturation of the response is correlated with a steady increase in the total number of mutations (Fig. 2). However, the scatter of binding affinities is large and the analysis of secondary and tertiary responses shows that the number of somatic mutations is not necessarily proportional to the increase in affinity. In spite of this, certain patterns of mutations are observable and this is particularly true in the V_κ-Ox1 gene. An accumulation of mutants in the CDR1/FRW2 border appears in the early stage of maturation of the response to pheOx (Table I). At later stages, additional mutations are more randomly distributed, although we noticed an accumulation of mutants at residue 92 of secondary and 50 of tertiary response V_κ-Ox1 L-

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Footnotes to Table III

The box indicates the observed pattern. * indicates patterns more likely than that observed.

Statistical analysis

Let there be N possible distinct silent changes.

Let there be M silent changes observed, made up of the pattern.

n_1 singletons
n_2 pairs
n_3 triples
n_4 quadruples of identical changes

Hence $M = n_1 + 2n_2 + 3n_3 + 4n_4$, and there are

$K = n_1 + n_2 + n_3 + n_4$ *distinct* changes.

Under the hypothesis that all changes occur at random (i.e. a probability 1/N of any particular change occurring), the probability of the *precise* pattern of changes occurring (i.e. the actual element changes that were observed) is the multinomal probability

$$\frac{M!}{1!n_1 2!n_2 3!n_3 4!n_4} \cdot \frac{1}{N^M} \qquad (1)$$

However, the particular set of N-K unobserved changes, n_1 singletons, n_2 pairs, etc. could be chosen from the N possible changes in

$$\frac{N!}{(N-K)!n_1!n_2!n_3!n_4!} \qquad (2)$$

different ways. Hence the overall probability of the observed pattern is expressions (1) × (2). For any particular pattern, the probability of observing a pattern at least as rare as the one observed can be used as 'p-value' measuring evidence against the hypothesis of random silent mutations, p = 1 – (sum of starred probabilities).

We are grateful to D. J. Spiegelhalter for these calculations.

chains (Fig. 2). A more careful examination of the pattern of mutations in the CDR1/FRW2 border emphasizes the non-random nature of the change. In almost all cases, the mutation involves the substituion of His 34 to Asn or Gln and of Tyr 36 to Phe. The importance of the mutation is highlighted by the fact that all tertiary response antibodies expressing the V_κ-Ox1 gene have mutated in this way (Figs. 2 and 4). Furthermore, other genes involved in late responses to phOx mutate to express the combination Asn/Gln 34 Phe 36 (Berek et al. 1985).

Chain recombination experiments using light chains, in which the different combinations of His, Asn or Gln at residue 34 and Tyr or Phe at 36 were the only differences, were made to assess their contribution to binding activity (Milstein et al. 1987). The results show that when residue 34 His is substituted for either Gln or Asn, the affinity constant increases by a factor of about 8 and 10 respectively (Table IV). This accounts for the bulk of the increase of the binding constant of NQ7/3.3 and of most of the increase of the best antibody of the d 14 response (NQ7/41.3). While selection must be a critical factor in the nature of the substitution, the efficiency with which the process takes place is remarkable. All mutations in the V_κ-Ox1 L-chains of the six hybridoma lines of the d 14 response expressing V_κ-Ox1/V_H-Ox1 are in the border region of CDR1/FRW2, including the characteristic change at residue 34. (This change is not seen in the two d 14 antibodies where V_κ-Ox1 is in association with other heavy chains). The mutations at residue 34 must have originated independently, because those antibodies differ

TABLE IV

The effect of a single substitution at residue 34 of the V_κ-Ox1 in the affinity for phOx

H/L chain reassociated molecules				
Hybridoma NQ		V_κ-Ox1 residue		Kd
Heavy chain	Light chain	34	36	10^{-7}M
2/16.2	2/16.2	H	Y	3.0
	21/12	H	F	2.3
	7/3.3	Q	F	0.6
	7/41.3	N	F	0.3
7/41.3	2/16.2	H	Y	1.9
	21/12	H	F	1.4
	7/3.3	Q	F	0.3
	7/41.3	N	F	<0.2
21/12	21/12	H	F	0.5
7/3.3	7/3.3	Q	F	0.7

Hybridoma NQ2/16.2 expresses the prototype V_HOx1/V_κOx1 anti phOx germline sequence. The light chains of the other hybridomas were chosen because they differ *only* at residues 34 and 36 as shown. The isolated heavy and light chains were separated and allowed to recombine by a small modification to the procedure described by de Preval & Fougereau (1976). The dissociation constant of the purified reassociated molecules was measured by fluorescence quenching.

from each other, either in residue 34 itself, or in the J- and/or D-segment. Indeed, the results summarised in Table I show that it is most unlikely for any of those lines to be clonally related.

The random probability that a single point mutation anywhere in the V_κ segment can generate either Gln or Asn at position 34 is around 1/500. In other words, if hypermutation starts in a synchronous manner after d 7, involving even as few as 10^4 cells, one would expect in a single division just over 20 cells to contain the mutation, assuming a mutation rate of 10^{-3}/base pair/generation, and 2 cells if the rate is 10^{-4}. These mutation rates apply to the specific bases involved and do not need to coincide with the average mutation rate for all bases of the V_κ-Ox1 light chain. This simple calculation is intended to show the type of mutation rates and the efficiency of the selection process which seems requisite to explain the remarkable pattern observed at d 14, as it applies to the His → Asn/Gln mutation.

The above arguments rely on the selective advantage of higher affinity for antigen. Such arguments are not directly applicable to the Tyr/Phe substitution at residue 36, since this does not affect binding affinity (see Table IV). What makes this substitution particularly intriguing is that residue 36 is an important H/L contact residue buried deep in the molecule (Poljak et al. 1975, Clothia et al. 1985). Tyr 36 is highly conserved among all known light chains, and only rarely substituted by residues other than Phe (Kabat et al. 1983). Such a substitution is unlikely to introduce important changes of conformation, but minor changes are difficult to predict. On the other hand, the base which mutates is in the middle of an extended palindrome corresponding to the sequence Trp.Tyr.Gln (Table I). These are all conserved residues which may not easily tolerate other substitutions, except silent ones, which are indeed detected (e.g. residue 37, see Fig. 4).

MUTATIONAL DRIFT AND REPERTOIRE SHIFT

Rearrangement of different germline gene segments V, D, J for the H-chain and V and J for the L-chain generates a broad spectrum of antibodies having various affinities for the hapten phOx. Out of this repertoire, the response to antigen begins. The earliest antibodies, usually of the IgM class, have a rather low average affinity for the hapten and express a complex array of V_H-D-J and V_κ-J segments (Kaartinen et al. 1986). The first step in the maturation of the immune response to phOx is the selection among those B cell clones of a restricted group expressing certain V_H-D-J and V_κ-J combinations to proliferate and differentiate. At this stage, the population will be biased by cells expressing germline gene combinations where good affinity coincides with high frequency. By d 7, the response to phOx is dominated by antibodies of the IgG1 class, and strongly biased to a given V_H-D-J and V_κ-J combination (V_H/V_κ-Ox1). The next stage of the maturation is dominated by the hypermutation process which initiates the genetic drift

of stimulated clones. Some of them are of high frequency (point mutants perhaps associated with hot spots) and those which improve recognition for antigen are quickly selected. A clear example has been discussed in the previous section. Further improvements by mutational drift become more difficult to achieve (i.e. may require multiple point mutations) with the V_κ-Ox1-V_H-Ox1 gene combination.

Other genetic combinations now begin to appear which at previous stages were of lower frequency. This could be the case with the V_H-MOPC21-like (V_H-M21) V_κ-Ox1 combination, which is expressed in several secondary and tertiary response antibodies. It is possible that the lower frequency of such B-cell clones at early stages is due to a low frequency of integration of the appropriate gene fragments or unusual generation of certain junctional variants during integration. For instance, the presence of Asp at position 95 of heavy chains expressing V_H-M21 may be important for phOx binding, but generated as an uncommon junctional diversity variant. Other gene combinations may have acquired anti-phOx specificity by alternative routes, for instance through a single mutation which by chance produced a good affinity product. These alternatives give rise to the shift in the germline gene combinations expressed in the oxazolone repertoire of the secondary and later responses.

The mechanism responsible for the interplay between mutational drift and repertoire shift is a fascinating property of the control of cellular proliferation during the immune response. Affinity for antigens is no doubt one important component of this complex puzzle, but is most unlikely to be the only one. Idiotypic interactions and, in particular, idiotypic suppression could be a way of dampening a dominating monoclonal response to favor the shift. Equally effective could be the compartmentalization of the response of virgin, primary and secondary cells. The role of germinal centers and of antigen-presenting cells could be crucial in this context. Lymphocyte migration and the life-span of virgin and memory cells could equally have important consequences for the outcome of our experiments.

Whichever are the processes which control and regulate the operation of the system, the consequences are clear. The system is *efficient* in delivering a fast response, even though this need not be of particularly high affinity. The system is *fast* in searching for improved alternatives, even though these may not initially be the best possible. The system is *thorough*, because it finds a way of continuously scanning the total repertoire in the search for even better alternatives.

CONCLUSIONS

The maturation of the immune response is the result of hypermutation and antigenic selection of variants of higher affinity. The hypermutation process is switched on *after* the initial antigen-induced proliferation of B cells and at about

the time when germinal centers are organized. The hypermutation process affects framework residues and complementarity-determining residues and introduces both silent and expressed mutations. However, the mutational drift is not fully random and certain positions are clearly more mutable than others. Based on the accumulation of silent mutation over a 7 d period of the early maturation of the response to phenyloxazolone, we calculate that the rate of hypermutation reaches values of the order of 10^{-3}/base pair/generation.

We have shown that a single nucleotide exchange in the V_κ-Ox1 gene is responsible for the major increase in antibody affinity during the early stages of the maturation of the response to phOx. The selective pressure is such that most, if not all, the mature antibodies expressing the V_κ-Ox1 gene express the mutant phenotype.

The selection of the proliferating pool is not restricted to high affinity antibody-producing cells only. Alternative gene combinations which may have started as low probability events, or later in the maturation of the response, become increasingly important as the maturation proceeds. This leads to a shift in the repertoire of the predominating germline gene combinations. The interplay between mutational drift and repertoire shift which ensures the continuous emergence of better antibodies is likely to depend on complex cellular interactions and other dynamic properties of the system.

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